AD_____

Award Number: W81XWH-06-1-0527

TITLE: Establishment of an “In Vitro Cell-Based System” to Assay Radiation Sensitivity in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Gregory T. Langland

CONTRACTING ORGANIZATION: University of California
Berkeley, CA 94720

REPORT DATE: May 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2007	Annual	1 May 2006 - 30 Apr 2007

4. TITLE AND SUBTITLE
Breast Tumor Establishment of an 'In Vitro Cell-Based System' To Assay Radiation Sensitivity in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0527

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Gregory T. Langland

E-Mail: GLangland@lbl.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Berkeley, CA 94720

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

14. ABSTRACT The purpose of this proposal is to develop an 'in vitro cell-based system' to study radio-resistance in sporadic breast cancer. By performing colony formation assays in response to X-ray treatment we have shown that there is a wide range of sensitivities to this form of treatment. Interestingly, we have also observed that tp53 status does not accurately predict response to radiation treatment. We have sensitized one of our most radio-resistant breast cancer cell lines to X-ray treatment by using RNAi-based technology to inhibit Artemis function. In the future, we feel these observations will have a significant clinical impact on the way radiation oncologists treat breast cancer patients.

15. SUBJECT TERMS
Breast cancer, cell culture, radio-sensitivity/resistance, RNAi, DNA repair, X-ray treatment

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Research Objectives:
The overall goal of this research project is to develop an *in vitro* cell culture system to assess the relative radiation sensitivity in a panel of breast cancer cell lines in order to develop a High Throughput System to identify novel targets using RNAi technology that will further sensitize these cells to X-ray treatment.

Introduction:

It is estimated that over fifty percent of women each year diagnosed with breast cancer will receive radiation treatment. However, it is often not known if the tumors will respond to radio or chemotherapeutic treatment. Radio-therapy also has many side effects that directly affect quality of life such as skin scarring, hair/pigment loss, chronic pain and fatigue. In order to help treat breast cancer patients and give radiation-oncologists a more detailed model of the response of sporadic breast tumors to radiation treatment we proposed to develop an *in vitro* cell culture system that accurately mimics the response of sporadic breast tumors to radiation treatment.

In order to understand how breast tumors respond to radiation it is important to address how radiation works and how breast epithelial cells respond to this treatment. X-rays are energy transmitted in the form of electrons that pass through the cell and cause damage to the DNA in the form of double strand breaks along with other forms of DNA damage. In a normal cell, there are stress response pathways and DNA repair pathways that are activated independent of each other. It is well known that *tp53* is responsible for activating genes that stall the cell cycle to allow the DNA to be repaired before entering back into the cell cycle. While this cell is stalled in G_1 or G_0 the predominant DNA repair pathway in activated is the non-homologous end-joining system is directly activated to coordinate the religating of the DNA double-strand breaks. This pathway is considered somewhat error-prone and a few nucleotides of sequence may be lost in this process. To add another layer of complexity to this field, the response of cells to X-rays is at least partially cell-type specific. Some cell-types such as lymphoblasts are very sensitive to X-ray exposure and quickly undergo apoptosis. While epithelial cells do not just enter an apoptotic pathway in response to radiation, but are also known to senesce and undergo mitotic catastrophe. Due to the heterogeneity of sporadic breast cancer genomes many of the stress response and DNA repair pathways are either non-functional or aberrant and this would suggest that there will be a range of responses to X-ray treatment.

Results:

Development of an '*in vitro* breast cancer cell line system'

Breast Cancer Cell Line Collection:
In order to establish an *in vitro* breast cancer cell line system that mimics sporadic breast tumors it is important to properly collect and maintain these lines. All breast cancer cell lines being used in this study were obtained directly from the Gray laboratory or purchased from ATCC (Table 1) and were cultured according to Neve *et al*.[1]. Once in culture, these lines were initially expanded to provide at least nine individual frozen stocks of early passage number cells. The breast cancer cell lines are also systematically tested for mycoplasma infection. The cell lines are being assayed in sets of twelve to keep all experiments well organized and cell cultures properly maintained.

Aim 1: To determine the relative IR-sensitivity of 55 human breast tumor and DCIS-derived cell lines as well as normal HMEC cultures.

Measuring the relative radio-sensitivity of the breast cancer cell lines:

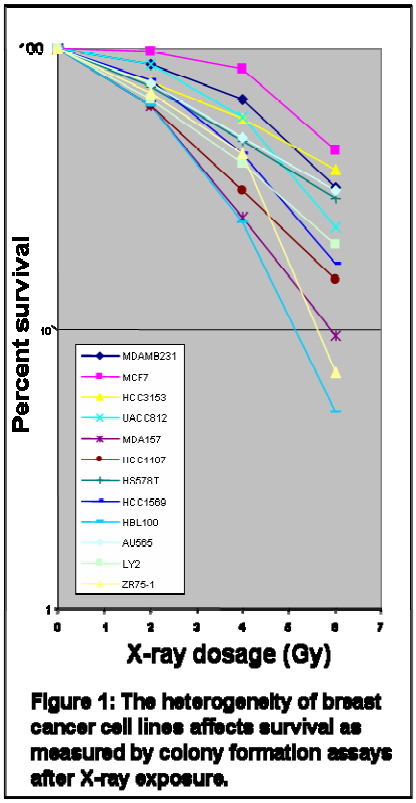

Figure 1: The heterogeneity of breast cancer cell lines affects survival as measured by colony formation assays after X-ray exposure.

Determining the relative radiation sensitivity can be quite complicated by the fact that there is no one simple ideal assay. We are determining the relative X-ray sensitivities of our panel of breast cancer cell lines by performing the traditional colony formation assays and/or by performing a BrDu FACS-based proliferation assay (**Figure 1**). We have also determined that a small percentage of our breast cancer cell lines do not readily form colonies *in vitro* dues to their inherent growth characteristics. The results presented in Figure 1 clearly show there are differences in how each breast cancer cell line responds to X-ray exposure. Careful examination of these data reveals that there is only a two-fold difference in the LD_{50} from the most-radio-resistant cell line in this panel, MCF7 and the most radio-sensitive line HCC1569. However, examination of **Figure 1** suggests that there may greater differences (more than 2 fold) in the LD_{90s} of these cell lines, since the LD_{90} of many of the cell lines tested can not be calculated because they were not exposed to a high enough dose to incur a 90% reduction in colony formation. These experiments are being repeated with higher doses of X-rays (eight and ten Gy) to provide accurate LD_{90} calculations. This will be much more informative to radiation oncologists since they want 100% tumor death not 50%. **Figure 2** shows the relative radiation sensitivity based on LD_{50} calculations and unlike our ovarian cancer studies were *tp53* status can be an accurate predictor of radiation sensitivity/resistance our initial observations suggest that *tp53* status does not strongly correlate with radiation sensitivity/resistance. Even though both of these cancers are derived from epithelial cell types these two different forms of cancer behave very differently when exposed to radiation.

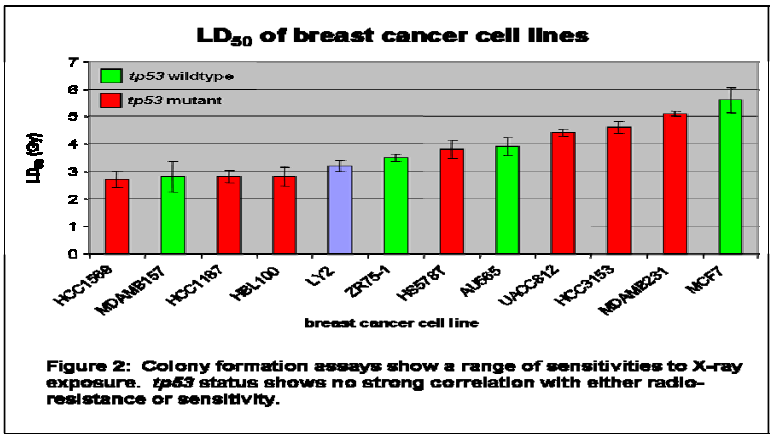

Figure 2: Colony formation assays show a range of sensitivities to X-ray exposure. *tp53* status shows no strong correlation with either radio-resistance or sensitivity.

Determination the *tp53* status of the breast cancer cell lines:

We have also addressed the *tp53* status of these cell lines, by using both bioinformatic and biochemical methods (**Table 1**). Utilizing the Welcome Trust Sanger Cancer Cell database, many of these cell lines' *tp53* exons

Cell #	Cell Line	Source	Classification	*tp53* exon seq.	tp53 status-stabilized protein	tp53 status
1	AU565	Gray Laboratory	Lu	WT	no	WT
2	BT20	ATCC	BaA	MUTANT	yes	MUTANT
3	BT474	ATCC	Lu	MUTANT	no	MUTANT
4	BT549	ATCC	BaB	MUTANT	yes	MUTANT
5	HBL100	Gray Laboratory	BaB	MUTANT	no	MUTANT
6	HCC1143	ATCC	BaA	WT	no	WT
7	HCC1187	Gray Laboratory	BaA	MUTANT	yes	MUTANT
8	HCC1500	Gray Laboratory	BaB	NOT DETERMINED	no	to be determined
9	HCC1569	Gray Laboratory	BaA	MUTANT	yes	MUTANT
10	HCC1937	ATCC	BaA	heterozygous mut.	yes	MUTANT
11	HCC3153	Gray Laboratory	BaA	heterozygous mut.	no	MUTANT
12	HS578T	Gray Laboratory	BaB	MUTANT	yes	MUTANT
13	LY2	Gray Laboratory	Lu	NOT DETERMINED	no	to be determined
14	MCF7	ATCC	Lu	WT	no	WT
15	MDAMB157	Gray Laboratory	BaB	WT	no	WT
16	MDAMB175II	Gray Laboratory	Lu	MUTANT	yes	MUTANT
17	MDAMB231	Gray Laboratory	BaB	WT	no	WT
18	MDAMB361	Gray Laboratory	Lu	MUTANT	yes	MUTANT
19	MDAMB453	Gray Laboratory	Lu	WT	no	WT
20	T47D	ATCC	Lu	heterozygous mut.	yes	MUTANT
21	UACC812	Gray Laboratory	Lu	MUTANT	yes	MUTANT
22	ZR75-1	Gray Laboratory	Lu	WT	no	WT
23	ZR7530	ATCC	Lu	MUTANT	yes	MUTANT
24	ZR75B	Gray Laboratory	Lu	WT	no	WT

have been sequenced. We have also compared these results with examining the steady state levels of tp53 protein since many *tp53* mutations result in an accumulation of non-functional cytoplasmic protein. A small percentage of the breast cancer cell lines *tp53* exons have not been sequenced by the Sanger Center and we are currently performing Q-PCR to measure radiation-induced *p21* transcript induction. As can be clearly seen by our data unlike our studies in ovarian cancer cell lines, several of the most radio-resistant breast cancer lines have no mutations detected in *tp53*. Suggesting, at least *in vitro tp53* functional does not correlate with relative radiation sensitivity/resistance. We are now pursuing the use of PAM-based analysis to identify other genes that are associated with radio-resistance.

Aim 2: To utilize RNAibased methodologies in order to specifically knock-down key NHEJ genes (Artemis or DNA-PKcs) to increase radiation sensitivity of selected breast cancer cell lines and normal HMEC cultures.

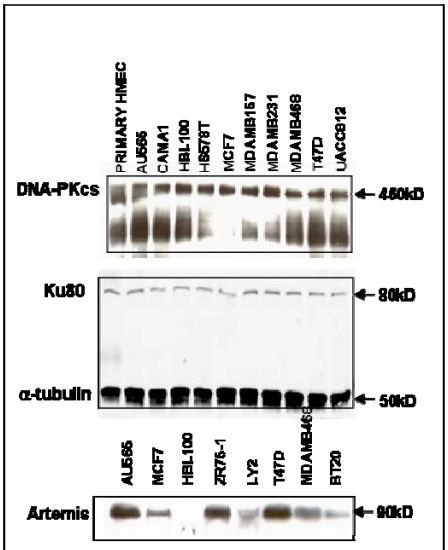

Figure 3: Western blot analysis of NHEJ proteins (DNA-PKcs, Ku80 and Artemis) showing relative protein levels of a subset of the breast cancer cell lines.

RNAi of selected NHEJ genes-Artemis and DNA-PKcs:

We have also made considerable progress in **Aim 2** of this project. The purpose of this aim is to develop RNAi-based strategies to knock-down either Artemis or DNA-PK to sensitize breast cancer cell lines to X-ray treatment. We were especially curious if the inhibition of Artemis will be a good radio-sensitizer, since this would be the first report using Artemis as a radio-sensitizing agent. Before we can begin knocking down these two proteins in breast cancer cells we have to establish the relative levels of these two proteins. Western blot analysis was performed on selected cell lines to visualize the levels of these two proteins. As seen in **Figure 3**, DNA-PKcs levels are not detectably different, however there is more variation in Artemis levels between the different breast cancer cell lines (data not shown). Inhibition of DNA-PK is known to sensitize cells as demonstrated by many different groups [2,3,4]. However, it is not known if the depletion of Artemis will sensitize breast cancer cells to radiation exposure. As a proof of principle we developed two lentiviral vectors that express small hairpin RNAs targeted against two different regions of the human Artemis transcript. The lenti vector expressing the shRNA against Artemis also contains a puromycin resistance gene in order to make stable cell lines expressing these shRNAs. As can be clearly seen in this western blot, our shRNAs reduce the relative amounts of Artemis protein as compared to the parental MDAMB231

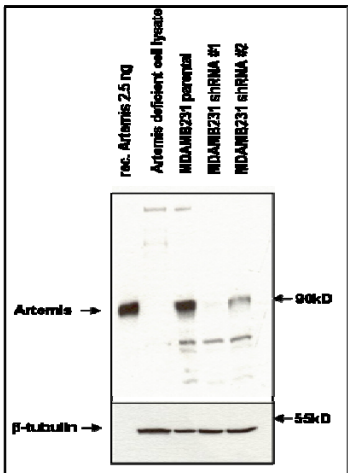

Figure 4: Lentiviral shRNA inhibition of Artemis expression in MDAMB231 breast cancer cells.

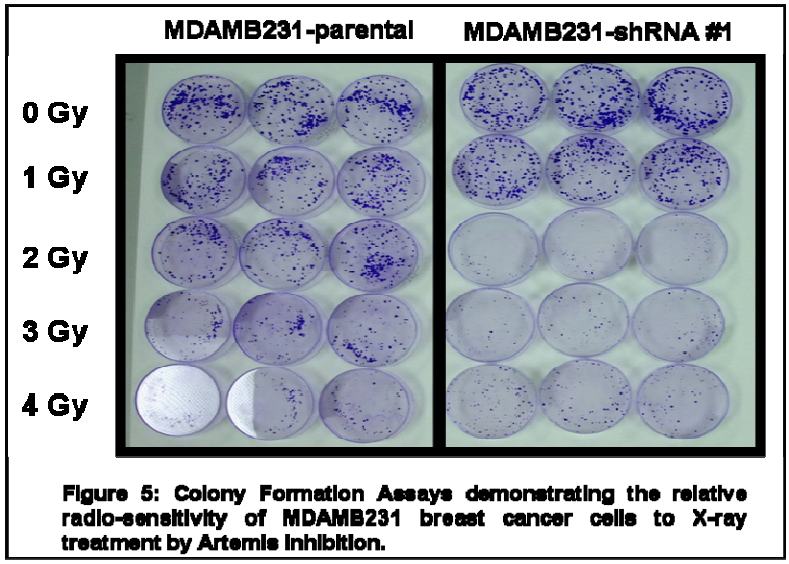

Figure 5: Colony Formation Assays demonstrating the relative radio-sensitivity of MDAMB231 breast cancer cells to X-ray treatment by Artemis inhibition.

cell line. When we performed survival assays, the MDAMB231 cell lines stably expressing the shRNAs and showing reduced levels of Artemis are much more sensitive to X-ray treatment. These results are not surprising since Artemis-deficient cells are more sensitive to X-ray exposure compared to control cells.

In order to create a HTP cell culture system we are currently optimizing the use of small interfering RNAs (siRNAs) directed against Artemis and DNA-PKcs. We will be using the Cellomics imaging system to utilize 96 well plate technologies to quickly assay the response of the breast cancer cell lines to X-ray treatment under siRNA inhibition. We will measure proliferation by staining for BrDu incorporation to determine a proliferating cell while counting the total number of cells by Hoechst staining. The counting is performed by their proprietary software with modifications made by members of the Gray laboratory. This software makes the analysis faster, more objective and most importantly quantitative.

Key Research Accomplishments:

We have established a panel of varied breast cancer cell lines necessary to measure relative radio-sensitivity. Multiple frozen stocks from early passage breast cancer cell lines have been generated along with total RNA samples as well as protein cell lysates. *tp53* status has been addressed by using sequencing information from the Sanger Centre and directly examining steady state levels of tp53 protein. Survival assays were performed on subsets of these breast cancer cell lines using traditional colony formation assays and by performing FACS-based proliferation assays based on BrDu incorporation. We have completed our first set of twelve breast cancer cell lines by CFAs. The second set is underway and these experiments will be completed by the end of July 2007. The relative amounts of several DNA repair proteins have been analyzed by Western blot analysis. We have also generated two lentiviral constructs that express shRNAs directed at the Artemis transcript. These lentiviral vectors contain a selectable marker (Puromycin gene) that allows the selection of clones that constitutively express the shRNA. Infection and selection of these vectors in MDAMB231 breast cancer cells results in the decreased expression of the Artemis protein. Survival assays have been performed on these cell lines and show a marked increase in radio-sensitivity when Artemis is depleted.

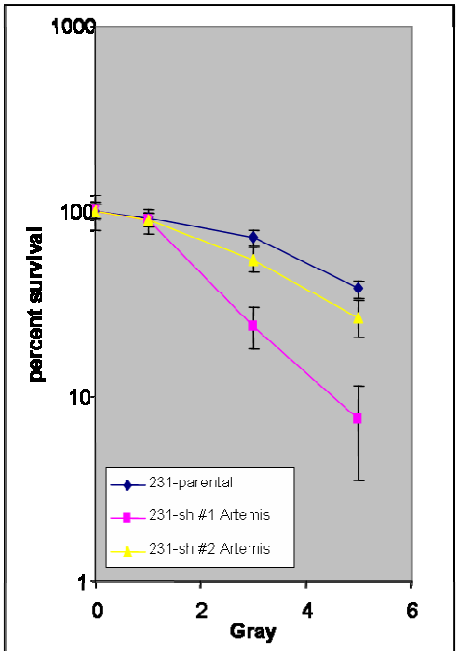

Figure 6: Percent survival of parental MDAMB231 breast cancer cell lines and with MDAMB231 expressing shRNAs directed at the Artemis transcript.

Reportable Outcomes:

By utilizing colony formation assays and proliferation assays we have detected significant differences of the breast cancer lines by measuring survival after X-ray exposure. These differences do not correlate with cell type classification, *tp53* status or overall growth rates. We have measured the relative amounts of various NHEJ proteins and most are relatively uniform but we have observed measurable differences in the steady state levels of the Artemis protein. Artemis may be a predictor of radiation sensitivity/resistance, but these initial observations need to be confirmed. However, the most exciting reportable outcome is the sensitization of the radio-resistant breast cancer cell line MDAMB231 by the shRNA inhibition of Artemis, since this observation has not been reported in the scientific literature.

Conclusions:

By performing survival assays in a collection of breast cancer cell lines we have established that there are measurable differences in the survival rates in response to X-ray treatment. We feel that these data will be beneficial to radiation oncologists in order to demonstrate that not all breast cancer respond in a similar manner to X-ray exposure. We are currently sensitizing some of the most radio-resistant breast cancer cell lines by employing novel RNAi technologies. Lentiviral shRNA knockdown has been shown to be very efficient at eliminating expression of Artemis and has been utilized by other groups but vector construction, shRNA viral production and subsequent characterization can be very time consuming. Therefore, much more of our efforts for the next year of this project will be devoted to the development of siRNAs' use as a potential therapeutic to sensitize the breast cancer cell lines to radiation treatment. By using siRNAs and the Cellomics Kineticscan imager we will be able to screen many more potential targets and more quickly determine which subsets of breast cancer cell lines will respond to these novel forms of treatment.

Materials and Methods:

Cell Culture:
All breast cancer cell lines were kindly provided by the Gray Laboratory or purchased directly from ATCC. Fetal bovine serum, basal medium, trypsin-EDTA and phosphate buffered saline were purchased from JRS Scientific. The cells were cultured at 37° C, 90% humidity with 5% CO_2.

Colony survival assays:
Cells were de-attached from their dishes by trypsin incubation and counted using a Coulter counter. For each X-ray dose, cells were plated at both 1000 and 5000 cells per well in triplicate. The cells were allowed to attach overnight and were exposed to X-rays using a Pantek X-ray machine at 320kV/10mA. The doses given were 0, 2, 4 and 6 Gy. The cells were placed in the incubator and incubated for ten to fifteen days to allow colonies to form. The colonies were then examined using phase-contrast microscopy to determine if there were approximately fifty cells per colony. The colonies were fixed and stained with a crystal violet solution and counted by hand. The percent survival was calculated using Microsoft Excel and curves subsequently plotted in semi-log scale. To calculate the LD_{50} of each of the cell lines the statistical analysis program Graphpad™ was used.

Lentiviral small hairpinRNA (shRNA) construct generation:
In order to efficiently knockdown Artemis expression shRNAs were designed according to standard algorithms. The oligos were synthezied by Operon technologies, allowed to anneal and cloned into a modified pENTRY vector. The pENTRY vectors were sequenced to verify the sequence of the shRNAs and propagated in the *E. coli* strain STBL3. LR reactions were performed according to the manufacturer's protocol (Invitrogen) and this allows the transfer of the shRNA into the Lenti-destination vector. These vectors were transfected into HEK293FT cells along with the helper plasmids necessary for lentiviral production. Lentiviral particles were purified using standard centrifugation techniques and stored at -80° C.

Selection of MDAMB231 cells expressing the shRNAs directed against Artemis:
MDAMB231 cells were infected with lentivirus for forty eight hours and then placed under antibiotic selection for two weeks. The stable clones are then analyzed for Artemis expression or lack there of by western blot analysis. Survival assays were performed on these cell lines according to the methods stated previously.

References:

1. Neve R, Chin K, Fridlyand J, Yeh J, Baehner F, Fevr T, Clark L, Bayani N, Coppe J, Tong F, Speed T, Spellman P, DeVries S, Lapuk A, Wang N, Kuo W, Stilwell J, Pinkel D, Albertson D, Waldman F, McCormick F, Dickson R, Johnson M, Lippman M, Ethier S, Gazdar A and J Gray A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006 Dec; 10(6):515-27.

2. Peng Y, Zhang Q, Nagasawa H, Okayasu R, Liber H, Bedford J. Silencing expression of the catalytic subunit of DNA-dependent protein kinase by small interfering RNA sensitizes human cells for radiation-induced chromosome damage, cell killing, and mutation. Cancer research. 2002 Nov 15;62 (22):6400-4.

3. Collis S, Schwartz M, Nelson W, DeWeese T. Enhanced radiation and chemotherapy-mediated cell killing of human cancer cells by small interfering RNA silencing of DNA repair factors. Cancer Research. 2003 Apr 1;63 (7):1550-4.

4. Cowell I, Durkacz B, Tilby M. Sensitization of breast carcinoma cells to ionizing radiation by small molecule inhibitors of DNA-dependent protein kinase and ataxia telangiectsia mutated. Biochemical Pharmacology. 2005 71:13-20.